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In Vitro* Stability of Phytase from Recombinant Bacteria *E. Coli BL21 (DE3) EAS1-AMP

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ABSTRACT

The objective of the research was to inquire the Km, Vm, activity, intracellular phytase stability exposed to pH variation, temperature variation and protease (pepsin and pancreas) *in vitro*. The phytase was produced from recombinant bacteria *E. coli BL21(DE3) EAS1-AMP* using 1.5 mM IPTG as inducer. Intracellular enzyme was extracted via freeze shock and centrifugation. Pure enzyme was acquired through NI-NTA agarose column. The enzyme was then tested for Km, Vm, phytase activity and stability against pH, temperature and protease. Treatment levels for stability against protease were P₀: without protease, P₁: addition of pepsin, P₂: addition of pepsin and pancreas, and the data were statistically analyzed using analysis of variance of one-way Completely Randomized Design. Crude intracellular phytase had Vm 6.39 μ M/sec, Km 34.82 μ M, and 277 units activity. Intracellular phytase was stable at pH 4–6 and 0–55^o C. Protease level influenced the activity of intracellular phytase (P<0.05). Intracellular phytase was stable against pepsin but not pancreas.

Keywords: Km, Vm, activity, intracellular phytase, pH, temperature, protease

Stabilitas Fitase Secara In Vitro dari Bakteri Rekombinan E. Coli BL21(DE3) EAS1-AMP

ABSTRAK

Penelitian bertujuan untuk mengetahui Km, Vm, aktivitas, stabilitas fitase intraseluler terhadap pH, suhu dan protease (pepsin dan pankreas) secara in vitro. Fitase diproduksi dari bakteri rekombinan E. coli BL21(DE3) EAS1-AMP dengan menggunakan induktor 1,5 mM IPTG. Ekstraksi enzim intraseluler menggunakan cekaman beku dan sentrifugasi. Enzim murni diperoleh dengan menggunakan kolom NI-NTA Agarosa. Enzim kemudian diuji untuk Km, Vm, aktivitas, serta stabilitas terhadap pH, suhu dan protease. Untuk stabilitas terhadap protease, level perlakuan adalah P₀: tanpa protease, P₁: penambahan pepsin, P₂: penambahan pepsin dan pancreas kemudian data dianalisis statistik menggunakan Rancangan Acak Lengkap Pola Searah. Fitase kasar intraseluler mempunyai nilai Vm 2,41 μ M/detik, Km 15,91 μ M dan aktivitas 246 unit. Fitase murni intraseluler mempunyai nilai Vm 6,39 μ M/detik, Km 34,82 μ M dan aktivitas 277 unit. Fitase intraseluler stabil pada pH 4 sampai 6 dan suhu 0 sampai 55^oC. Level protease berpengaruh terhadap aktivitas fitase intraseluler (P<0,05). Fitase intraseluler stabil terhadap pepsin, tetapi tidak stabil terhadap pankreas.

Kata kunci: Km, Vm, aktivitas, fitase intraseluler, pH, temperatur, pepsin, pankreas

INTRODUCTION

Phytase enzyme is absent in the digestive tract of non-ruminant animals since the digestive tract of non ruminant do not produce this enzyme. Therefore, phytates found in seed cannot be digested due to its strong chelating character and discarded to the environment with feces (Shint *et al.*, 2001) in the form of phytate bound (Jendza *et al.*, 2006). It is simultaneously excreted by poultry to the environment which can cause serious negative effects (Saryska *et al.*, 2005). This waste is a pollutant to the environment (Daniel *et al.*, 1998).

Phytase is an enzyme, which is also a protein, thus can be easily degraded by environmental conditions (Anselme, 2006). Some requirements which must be observed in the practical application of enzyme in poultry are that the enzyme must be able to withstand heat, acidic pH, and protease activity (Abondano, 2009).

Pure intracellular phytase from recombinant bacteria *E. coli* BL21(DE3) *EAS1-AMP* had been produced and characterized (Nuhriawangsa *et al.*, 2010). However, the stability of the enzyme in the digestive tract of non ruminant animal is not yet known, thus the research was aimed to determine the V_m , K_m , activity and intracellular phytase stability *in vitro*.

MATERIALS AND METHOD

Materials used in this experiment were recombinant bacteria *E. coli* BL21(DE3) *EAS1-AMP*, crude intracellular phytase for pH and temperature stability trial, pure intracellular phytase for protease level trial, Luria Bertany (LB) medium containing Amp 25 $\mu\text{g/ml}$, STOP solution, 1.5 mM IPTG, NI-NTA Agarose, Na-Phytate, rice brain, distilled water, HCl, pepsin (Sigma, catalog P-7012), NaHCO_3 , pancreas (Sigma, catalog P-3292), dialysis tube (Sigma, catalog D-9652-100FT), parafilm, NaCl, EDTA, sodium succinate, and sulphuric acid.

Measurement of K_m , V_m , and Phytase Activity

K_m and V_m values were determined following according to Robyt and White (1997) by comparing products before and after hydrolysis. Phytase activity value in unit enzymes, was defined as portion of phytase that can convert 1 μmol orthophosphate from phytate per minute at pH 5.5 and 37°C (Zyle *et al.*, 1995). In this research pH 5 and 45°C , the optimum intracellular phytase temperature and pH was used (Nuhriawangsa *et al.*, 2009).

Measurement of Phytase Relative Activity

Phytase activity was measured following the method of Sajidan (2002): 50 μl enzyme, 150 μl substrate (0.4% Na-phytate in 100 mM Na-acetate) incubated at 37°C for 30 minutes. Reaction was stopped by adding 400 μl STOP solution. Yellow color of phosphomolybdate was measured using spectrophotometer at λ 415 nm.

Analysis of Phytase pH and Temperature Stability

The pH and temperature stabilities were measured according to Sajidan (2002) by observing the relative activity. Temperature stability trial was done by heating the phytase at varying temperature (0, 20, 37, 40, 45, 50, 55, 60, and 65°C) for 10 minutes and followed by phytase activity trial. The pH stability trial was done by incubating phytase in Na-acetate buffer at varying pH (2, 3, 4, 5, 6, 7, 8).

Analysis of Phytase Stability against Protease

Treatment levels were as follows P_0 : without protease, P_1 : addition of pepsin (6000 units/ml), P_2 : addition of pepsin (6000 units/ml) and pancreas (3.7 mg/ml). Phytase activity was evaluated by P digestibility *in vitro* on rice brain, which was a modification of the crop, gizzard, duodenum and small

intestine according to Wu *et al.* (2004). *In vitro* digestibility was observed based on the results of P value trials using spectrophotometer at λ 415 nm (Jackson, 1985). Digestibility value P was calculated by observing the difference in P value of feed before and after *in vitro* analysis in dry matter condition (Tillman *et al.*, 1986).

Statistical Analysis

Statistical analysis of Km, Vm, enzyme activity, pH and temperature stability was conducted using discriminative quantitative analysis by observing the mean value from relative enzyme activity. Statistical analysis of protease stability was done by using analysis of variance of one way Completely Randomized Design (Steel and Torrie, 1993). Statistical analysis was carried out using Minitab 14 program for Microsoft Office 2003.

RESULTS AND DISCUSSIONS

The Vm, Km and activity values of pure and crude intracellular phytase are shown in Table 1. Results show that Vm, Km, and activity of pure intracellular phytase were better than crude intracellular phytase. Enzymes are proteins that possess catalytic activity due to its active group. Enzymes are produced after microbial colony is planted in media followed by fermentation. Crude enzyme after fermentation was separated from the media by ultrafiltration, precipitation, extraction, and other methods. Crude enzymes were then purified until a certain level of purity (Clarkson *et al.*, 2001). Enzyme purification can be conducted by dialysis or ion exchange chromatography (Raju *et al.*, 2007). According to David and Thomas (1990) protein purification is carried out with the purpose of removing contaminants so that quantitatively the protein is free from contaminants. The purified product will have better Vm, Km and enzyme activity because various inhibitors dissolved was removed from the solvent during enzyme purification.

Results of pH stability of intracellular phytase is shown on Figure 1. Phytase activity ranged between pH 2 and 8. Phytase activity increased at pH 2–4 and reached optimum level at pH 5, then decreased at pH 6–8. Phytase had low activity at pH 2, 7 and 8, but stable at pH 4–6. Enzyme activity was influenced by pH because at certain pH conditions the enzyme cannot be active due to denaturation or degradation (Hendrickx *et al.*, 1998). The pH influenced enzyme balance condition during denaturation, whether it is reversible or irreversible. Denaturation caused structural changes in the molecular structure of the enzyme protein, thus whether it remains active or becomes inactive will depend on the type of the damage, reversible or irreversible (Zale and Klivanov, 2004).

The bound of amino acid residues on protein are dependent on the proton concentration in its molecular composition. Increasing accumulation of pH value caused positif or negative instability in the molecular structure of enzyme protein. Structural instability in the enzyme protein molecule caused structural changes, leading to thus decreased of the enzyme's catalytic activity (Furlan and Pant, 2008).

The pH of the digestive tract of chicken in crop 4.5, proventriculus 4.4, gizzard 2.6, duodenum 5.7–6.0, jejunum 5.8, ileum 6.3, colon 6.3, and ceca 5.7 (Sun, 2004). Phytase from *E. coli* BL21 (DE3) EAS1-AMP recombinant bacteria was stable at pH 4–6. Thus, this phytase active in the crop, proventriculus, gizzard, duodenum, jejunum, ileum, colon, dan ceca, but its activity in the gizzard is low.

Results of intracellular phytase stability against temperature is shown in Figure 2. Increasing the temperature caused reduction in phytase activity. This reduction was apparent at temperatures of 55–66⁰ C. Phytase activity was low at 60⁰C and almost inactive at 65⁰C. According to Illanes (1994), the increased of temperature resulted in the increased of vibrational energy that broke the hydrogen bounds, thus the non-polar bound did not exist. This caused structural changes in

Table 1. The Vm (μM/sec), Km (μM) and phytase activity (Unit) of crude intracellular and pure intracellular from recombinant bacteria *E. coli BL21(DE3) EAS1-AMP*

| Type of Phytase | Vm | Km | Activity |
|---------------------|--------|---------|----------|
| Crude intracellular | 2,41* | 15,91* | 246 |
| Pure intracellular | 6,39** | 34,82** | 277 |

*Source: Nuhriawangsa (2009).

**Source: Nuhriawangsa (2010).

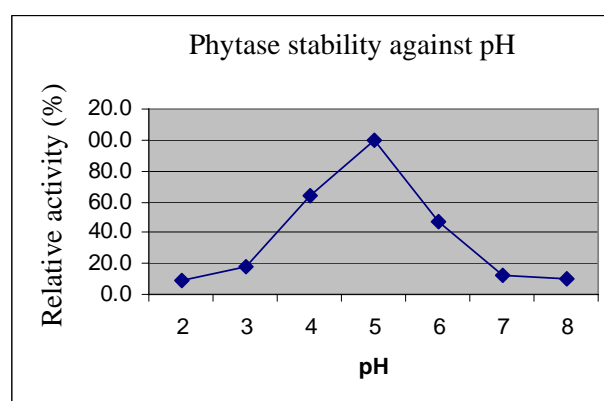


Figure 1. Relative activity of phytase (%) at variuos pH and temperature 45°C

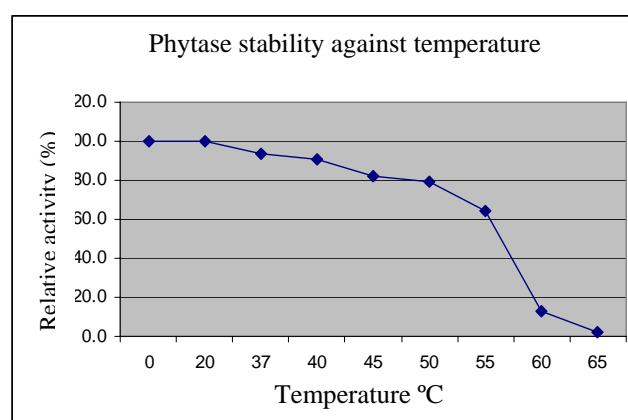


Figure 2. Relative activity of phytase (%) at various at temperature

enzyme protein molecular structure and affected the enzyme catalytic activity.

The reduction of activity was caused by phytase denaturation due to heat exposure (Furlan and Pant, 2008). Phytase *Thermomyces* denaturates at 66°C (Berka *et al.*, 1998). Phytase from *A. ficuum* at 70°C denaturates 25% at pH 2 and 65% at pH 8.5. (Shieh *et al.*, 1969).

The normal process of pellet production for poultry feed is conducted at 85–95°C, while the temperature of chickens' digestive tract is about 37°C. Phytase from *E. coli BL21 (DE3) EAS1-AMP* recombinant bacteria was stable at 0–55°C. Wang *et al.* (2007) found that phytase from *Aspergillus fumigatus* WY-2 had a low stability against high temperature, causing low activity due to enzyme damage.

Table 2. The average of P (g) content in rice bran (dry matter basis) after hydrolisis without the treatments of phytase (F1), with fitase (F2), without protease (P1), with pepsin (P2) and with pepsin and pancreas (P3)

| Phytase | F1 | F2 | Average [*] |
|----------|----------------------|----------------------|------------------------|
| Protease | | | Average ^{**} |
| P1 | 0.00335 | 0.00500 | 0.00418 ^x |
| P2 | 0.00431 | 0.00550 | 0.00490 ^{x,y} |
| P3 | 0.00187 | 0.00338 | 0.00263 ^z |
| Average | 0.00318 ^a | 0.00463 ^b | |

^{a,b} Different superscript in the same line indicating significantly differences (P<0.01).

^{x-z} Different superscript in the same column indicating significantly differences (P<0.01).

Wyss *et al.* (1999) also found that phytase from *Aspergillus fumigatus* was stable at low temperature. These studies resulted phytase with low stability against high temperature, thus only active in the digestive tract of chickens but cannot be applied during pellet production.

In vitro result of pure intracellular phytase in rice brain against protease stability by evaluating digestibility P is shown in Table 2. Addition of enzyme into feed needs specific requirement since the enzyme cannot be degraded by protease in the digestion tract (Garret *et al.*, 2004; Wang *et al.*, 2007). This due to the fact that the production of phytase by eco-friendly bacteria and stable against proteolytic activity is needed so that it can be applied to poultry feed (Igbasan *et al.*, 2000). Some research have been conducted to test the stability of phytase against pepsin and tripsin produced by poultry digestive organs (Wang *et al.*, 2007; Kornegay, 2001) and pancreatin (Igbasan *et al.*, 2000; Phillippy, 1999).

The P content of rice bran after *in vitro* hydrolysis was different (P<0.05) between without protease and with pepsin, without pepsin and combination of pepsin pancreas and pepsin with combination of pepsin pancreas. P content increased with the addition of pepsin and decreased with the addition of pepsin–pancrease combination. According to Kornegay (2001) the decrease in enzyme activity caused by degradation by proteolytic enzyme. Reduction in enzyme activity due to proteolytic enzyme degradation is an important matter, because the activity of

an enzyme against protease stability can be measured by observing the phytase activity against pepsin (Wang *et al.* 2007) and pancreas (Igbasan *et al.* 2000). Phytase from recombinant bacteria *E. coli* BL21 (DE3) *EAS1-AMP* was not hydrolyzed under the influence of pepsin, thus phytase activity was stable against pepsin, which was proven by increased of P content with the addition of pepsin compared to the absence of protease. In contrast, phytase was hydrolyzed by combination of pepsin and pancreas, which was shown by the decrease in P content at level of pepsin and pancreas, thus phytase was not stable against pancreas.

CONCLUSION

Crude intracellular phytase had Vm 2.41 μ M/second, Km 15.91 μ M and activity 246 units. Pure intracellular phytase had Vm 6.39 μ M/second, Km 34.82 μ M and activity 277 units. Intracellular phytase from recombinant bacteria *E. coli* BL21 (DE3) *EAS1-AMP* was stable at pH 4–6, 0–55⁰ C and pepsin, but was not stable against pancreas.

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